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The B cell coactivator Bob1 shows DNA sequence-dependent complex formation with Oct-1/Oct-2 factors, leading to differential promoter activation

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We have shown previously that both octamer binding transcription factors, namely the ubiquitous Oct-1 and the B cell-specific Oct-2A protein, can be enhanced in transcriptional activity by their association with the B cell-specific coactivator protein Bob1, also called OBF-1 or OCA-B. Here we study the structural requirements for ternary complex formation of DNA–Oct–Bob1 and coactivation function of Bob1. In analogy to DNA-bound transcription factors, Bob1 has a modular structure that includes an interaction domain (amino acids 1–65) and a C-terminal domain (amino acids 65–256), both important for transcriptional activation. A mutational analysis has resolved a region of seven amino acids (amino acids 26–32) in the N-terminus of Bob1 that are important for contacting the DNA binding POU domain of Oct-1 or Oct-2. In contrast to the viral coactivator VP16 (vmw65), which interacts with Oct-1 via the POU homeosubdomain, Bob1 association with Oct factors requires residues located in the POU-specific subdomain. Because the same residues are also involved in DNA recognition, we surmised that this association would affect the DNA binding specificity of the Oct–Bob1 complex compared with free Oct factors. While Oct-1 or Oct-2 bind to a large variety of octamer sequences, Bob1 ternary complex formation is indeed highly selective and occurs only in a subset of these sequences, leading to the differential coactivation of octamer-containing promoters. The results uncover a new level in selectivity that furthers our understanding in the regulation of cell type-specific gene expression.

Keywords: B-cell coactivators/DNA binding specificity/POU factors/protein–protein interaction/transcriptional activation

Introduction

Immunoglobulin gene expression is restricted to B cells through B cell-specific promoter and enhancer elements and their cognate transcription factors. A conserved *cis* element, the octamer motif (ATGCAAAT), is found in most of the Ig promoters and enhancers and seems to be a major determinant of the B cell-restricted expression of Ig genes (Kemler and Schaffner, 1990; Jenuwein and Grosschedl, 1991; Staudt and Lenardo, 1991). However, the same octamer motif is also critical for the promoters of ubiquitously expressed genes like the H2B gene (Sive *et al.*, 1986), the VP16-dependent immediate-early gene promoters of herpes simplex virus (apRhys *et al.*, 1989) and even for polymerase III promoters like the U6 promoter (Danzeiser *et al.*, 1993).

To date, several octamer binding transcription factors and their cDNAs have been isolated (for a review see Schöler, 1991). All of these octamer binding proteins belong to the POU family of DNA binding transcription factors, characterized by a bipartite DNA binding domain: the so-called POU domain. This domain consists of two independent DNA binding modules, the N-terminal POU-specific and the C-terminal POU homeosubdomain, separated by a flexible linker (Rosenfeld, 1991; Herr and Cleary, 1995). A large number of related factors have been cloned that seem to be important developmental regulators, but only the broadly expressed Oct-1 and lymphoid-specific Oct-2 can be found in B cells (Staudt *et al.*, 1986; Müller *et al.*, 1988).

The initial hypothesis, that the B cell-restricted Oct-2 protein accounts for the B cell specificity of octamer promoters (Müller *et al.*, 1988), was questioned by several observations (Pierani *et al.*, 1990; Corcoran *et al.*, 1993). The finding that *in vitro* transcription from an Igκ promoter activated by purified Oct-1 and Oct-2 can be enhanced further by the addition of a fraction from B cell nuclear extract called OCA-B (Pierani *et al.*, 1990; Luo *et al.*, 1992) supported the idea that an additional B cell-specific coactivator interacting with either Oct-1 or Oct-2 is required for the observed B cell specificity of octamer-containing promoters and enhancers (Annweiler *et al.*, 1992).

Recently we and others have isolated the cDNA encoding a 35 kDa B cell-specific transcriptional coactivator of Oct-1 and Oct-2, variously termed Bob1, OCA-B or OBF-1 (Gstaiger *et al.*, 1995; Luo and Roeder, 1995; Strubin *et al.*, 1995), which is the first example of a cell type-specific coactivator. This factor, for simplicity referred to here as Bob1, can enhance Oct factor-dependent transcription in non-B cells by virtue of its association with the POU domains of Oct-1 and Oct-2. The interaction with the POU domains of Oct-1 and Oct-2 is specific because other POU domain transcription factors, like Oct-3/4, Oct-6, N-Oct2 and N-Oct3, were not recognized by Bob1 (Gstaiger *et al.*, 1995; Strubin *et al.*, 1995). Bob1 cannot interact with the octamer motif itself (Luo and Roeder, 1995), and encodes a transcriptional activation domain which can confer transcriptional activity when fused to the DNA binding domain of GAL4 (Gstaiger *et al.*, 1995), indicating that coactivation can be explained, at least in part, by tethering an additional activation domain to the octamer promoter. However, this simple

view of Bob1 function would imply that any promoter recognized by Oct-1 or Oct-2 would be activated by Bob1.

Experiments with the octamer promoter of the ubiquitously expressed H2B gene showed no detectable activation *in vitro* (Luo and Roeder, 1995) and reduced coactivation levels *in vivo* compared with activation of the Igκ promoter in the presence of Bob1 (Strubin *et al.*, 1995). Because the H2B octamer element allows ternary complex formation with Bob1 that is indistinguishable from that on the Igκ octamer (Figure 4b and c), the reduced coactivation level of the H2B promoter indicates that the promoter context plays a role, at least to some extent, in specifying the degree to which Bob1 boosts Oct factor-dependent transcription. Alternatively, promoter-specific activation by the Oct–Bob1 complex could be achieved by simply altering the DNA binding specificity of the complexed versus the free Oct factor, since it is known that Bob1 interacts with the DNA binding domain of Oct-1 or Oct-2.

Here we show that Bob1 contains different functional domains. It consists of an N-terminal interaction and a C-terminal transcriptional activation domain. Point mutations within the interaction domain completely prevent interaction and coactivation function. Bob1 interaction with the Oct-1 POU domain requires residues located within or close to the DNA recognition helix of the POU-specific domain. This indicates that Bob1, although not directly contacting DNA, possibly influences DNA binding. This idea is supported by our finding that the Oct–Bob1 ternary complex formation occurs only with a subset of octamer sites. In agreement with this result, we find that Bob1 coactivation does not occur on all octamer sites bound by Oct-1. Rather, it is highly sensitive to slight changes within the octamer sequence. Taken together, these results show that Bob1 does not simply act as a cell type-specific amplifier of Oct factor-dependent transcription but, in addition, selectively confers its intrinsic transactivation capacity to specific octamer sites. This indicates a novel mechanism for the cell type-specific activation of octamer-containing promoters.

Results

The B cell-specific coactivator Bob1 can be dissected into functional domains for interaction and transactivation, similar to DNA-bound transcription factors

We have shown previously that tethering of the Bob1 protein to a promoter containing a GAL4 site by simply fusing the Bob1 cDNA to the DNA binding domain of GAL4 is sufficient for transactivation (Gstaiger *et al.*, 1995). The simplest interpretation of this is that coactivation in the context of the Oct-1–Bob1 and Oct-2–Bob1 complexes (referred to as Oct–Bob1 complexes) functions by bringing an additional transcriptional activation domain to the octamer site in the promoter, rather than merely inducing the unfolding of an intrinsic activation domain of the octamer factor. To clarify this issue, we analysed several deletion mutants of Bob1 for their interaction and coactivation properties. Figure 1a shows the result of the deletion analysis to identify the Oct-1 interaction domain of Bob1. To distinguish retarded ternary complexes containing Bob1 deletion mutants from the Oct-1 bandshift, we used an anti-FLAG antibody against Bob1 mutants, which were tagged with the FLAG epitope at the N-terminus and translated in reticulocyte lysates. The data show that all the C-terminal deletion mutants tested, including a deletion where only a region encoding the first N-terminal 65 amino acids of the Bob1 protein is expressed, are sufficient for contacting Oct-1. Removal of this region leads to a complete loss of interaction with Oct-1. The observed slight variations in supershift activity probably reflect differences in the amount of Bob1 protein in the reticulocyte lysates, as estimated from protein gels. We also tested the transactivation capacities of the same Bob1 deletion mutants by cotransfection into HeLa cells. Interestingly, interaction with the Oct-1 factor is not sufficient for coactivation because the C-terminal deletion mutant Bob1(1–65), which still binds Oct-1, is unable to transactivate (Figure 1b, lane 5), whereas the Bob1(1–122) mutant, where almost half of the protein is removed, shows a 90% coactivation level compared with wild-type Bob1 (Figure 1b, lane 4). When fused to the DNA binding domain of GAL4, both the regions from amino acids 65–122 and 65–256 of Bob1 activate a promoter with a GAL4 binding site ~3.3- and 3.0-fold, respectively, when compared with the GAL4 DNA binding domain alone (data not shown). This confirms that most of the transcriptional activation is exerted by the region between amino acids 65 and 122. However, in the context of Oct factor-dependent transcription, a minor contribution in coactivation activity by the region from amino acids 122 to 256 cannot be excluded at present. Not unexpectedly, the N-terminal deletion mutants no longer showed any coactivation (Figure 1b, lanes 6–8), indicating that a physical interaction with the octamer factor Oct-1 is required for transactivation by Bob1. Taken together, it appears that, in analogy to DNA-bound activators, the coactivator Bob1 also seems to be organized in at least two functional domains (Figure 1c): an N-terminal domain (amino acids 1–65) important for contacting Oct-1, and a C-terminal domain which functions as an activation domain in the native protein or when linked to the heterologous DNA binding domain of GAL4.

A stretch of seven amino acids located in the N-terminus of Bob1 is crucial for Oct-1/Oct-2 interaction

To analyse the structural basis of the Oct–Bob1 interaction, we selected various Bob1 mutants with altered interaction properties in a yeast two-hybrid screen (Gstaiger *et al.*, 1996). Random mutations were generated by degenerative PCR covering the region delineated by amino acids 1–122. We isolated four independent Bob1 point mutants from the yeast screen with reduced interaction abilities. Point mutations that prevent any interaction with Oct-1 or Oct-2 were located within a seven amino acid stretch in the N-terminus (amino acids 26–32) of the mutated region (Figure 2c). No mutants in the region from amino acids 65 to 122 outside of the interaction domain were selected, whereby mutant L32P was independently isolated twice.

To determine whether these mutants had the same phenotype in mammalian cells, we tested them for their interaction properties in electrophoretic mobility 'super-

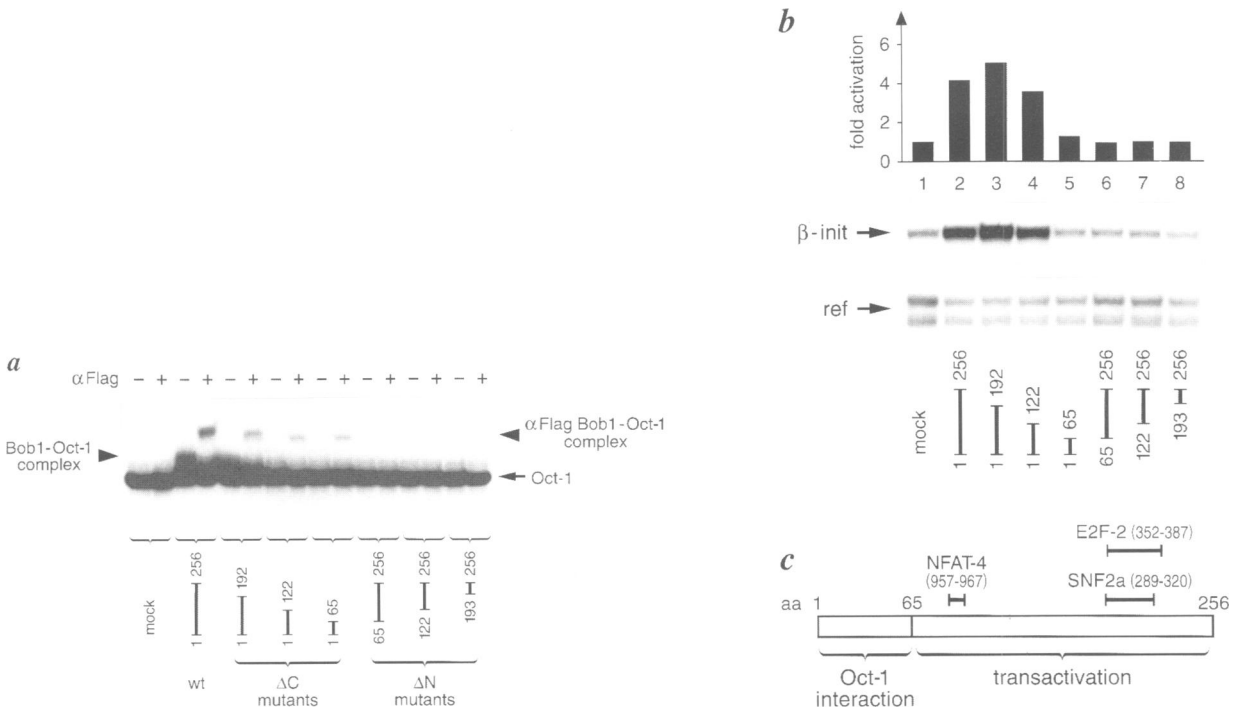

Fig. 1. Bob1 contains different functional domains. (**a**) The Oct-1 interaction domain is located within the first 65 amino acids of the N-terminus of Bob1. The gel retardation experiment shown was performed with different Bob1 deletion mutants expressed in rabbit reticulocyte lysates supplemented with Oct-1 from HeLa nuclear extract and with a radiolabelled Igκ octamer oligonucleotide. The presence or absence of a monoclonal antibody against the FLAG epitope-tagged Bob1 proteins is indicated by (+) and (–). Retarded bands representing different complexes are depicted, and the tested Bob1 mutants are drawn schematically beneath the autoradiogram. The numbers refer to the amino acid sequence of Bob1 expressed in the deletion mutants. (**b**) The transactivation function and Oct-1 interaction function of Bob1 are located in two separable protein domains. RNA from HeLa cells transfected with 5 μg β-globin reporter construct Igκ–OVECS containing the Igκ octamer site in the promoter, 0.5 μg reference plasmid OVEC–REF and 5 μg pCATCH plasmids expressing various Bob1 deletion mutants (for details of the expression constructs see text) was analysed in an S1 nuclease assay. The signals were quantified with a PhosphorImager. Relative transcription levels standardized for the signals derived from the cotransfected reference gene OVEC–REF are depicted in the diagram above. 'β-init' indicates the signal from the β-globin reporter gene; 'ref' marks the signal of reference gene. (**c**) Schematic illustration of the functional domains of the Bob1 coactivator. Numbers indicate the amino acid position within the Bob1 sequence which mark the borders of the functional domains, as defined by the deletion analysis. Regions of protein sequence identity (Bob1 amino acids 85–95 to NFAT-4, 54% identity; Bob1 amino acids 187–212 to SNF2a, 46% identity; Bob1 amino acids 187–222 to E2F-2, 36% identity) were found using the BLAST program (Altschul *et al.*, 1990). The numbers in parentheses shown in the figure refer to the amino acid positions of the corresponding proteins.

shift' experiments and subsequently analysed them for the transactivation of Oct factor-dependent transcription in human 293T cells. The higher expression levels in 293T cells compared with HeLa cells allowed us to simultaneously measure interaction capacity and coactivation levels of the Bob1 mutants from the same transfection experiment. As shown in Figure 2a, Bob1 mutants E26G+P27L, E30L and L32P, when expressed in 293T cells, do not interact with Oct-1 under our bandshift conditions, although they were expressed to similar levels as estimated by a Western blot analysis (data not shown). Another Bob1 mutation (V51E), which is located outside of this seven amino acid region, was unaffected in its interaction with Oct-1. This mutant was initially isolated as a loss-of-function mutant in the two-hybrid mutagenesis screen because of an additional frame shift mutation 5′ of the Bob1 coding region in the fusion protein. When we tested these mutants for coactivation, we found that all of the point mutants which did not supershift also showed a drastic reduction in coactivation function (Figure 2b). Mutant E30L, although unable to interact stably in our bandshift experiments, was still able to coactivate to a low extent when overexpressed in 293T cells.

Bob1 interaction with the Oct-1 POU domain requires residues located in or close to the DNA recognition helix of the POU-specific domain

A number of proteins have been demonstrated to bind the POU homeodomain, including VP16 from herpes simplex virus, the viral precursor terminator DNA polymerase complex (pTP–Pol) from adenovirus, and cellular HMG2 protein (Stern *et al.*, 1989; Coenjaerts *et al.*, 1994; Zwilling *et al.*, 1995). Oct-1 and Oct-2A are 99% identical in their POU-specific domains, which is significantly higher than in the homeodomain (88%). Given the fact that the Bob1 interaction is highly specific for Oct-1 and Oct-2, we reasoned that the interaction is mediated by the POU-specific domain of the two Oct factors. A series of bandshift experiments using bacterially produced mutant proteins of the Oct-1 POU domain, together with recombinant Bob1, allowed us to study the structural basis of the observed interaction between Bob1 and the POU domains. First we analysed a series of point mutants within surface-exposed residues of the Oct-1 POU-specific domain for their capacity to interact with Bob1 (Figure 3a). Point mutants L32A+K36A, S43A, Q44A, T45A, S48A and N54A showed an interaction capacity with

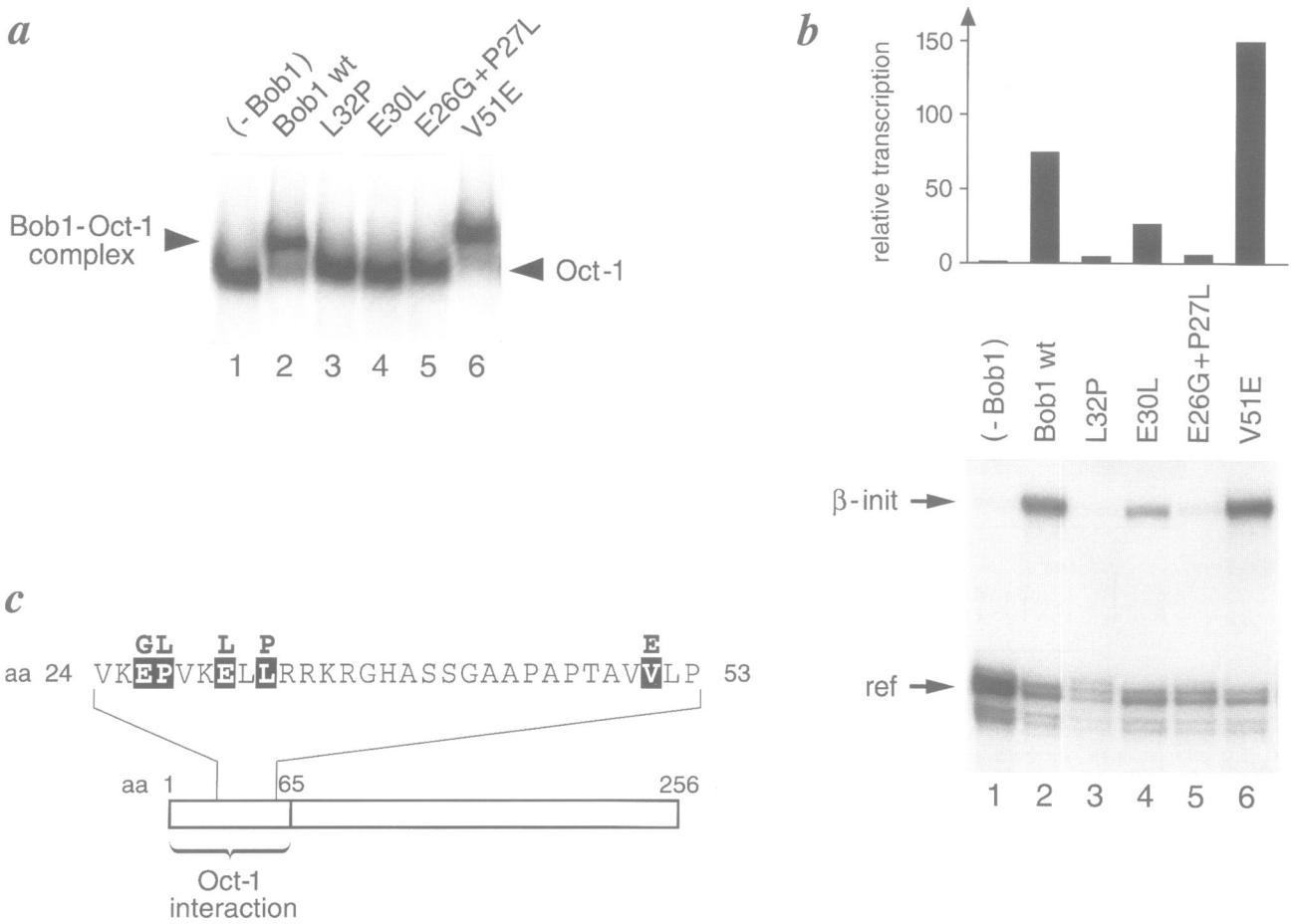

Fig. 2. Bob1 point mutations with impaired interaction and coactivation function. 293T cells were transfected with 5 μg CMV vector pCATCH–NLS for expression of the indicated Bob1 mutants, 5 μg reporter plasmid Igκ–OVECS and 0.5 μg reference plasmid CMV–REF. The nuclear extract preparation and the S1 nuclease assay were performed from the same transfection. (**a**) Bob1 mutants tested for Oct-1 interaction. Aliquots (5 μl) of nuclear extract from transfected 293T cells were incubated with radiolabelled Igκ octamer oligonucleotide and analysed for 'supershift' activity. The expression of the tested Bob1 mutants was confirmed by a Western blot analysis (data not shown). (**b**) An S1 analysis of transfected 293T cells. Relative transcription levels are shown in the diagram above the autoradiogram and were calculated as indicated in the legend to Figure 1. (**c**) Schematic representation of the Bob1 mutations tested. Amino acids are given in one-letter code and the numbers refer to the position within the Bob1 amino acid sequence.

Bob1 comparable with wild-type Oct-1 POU. Mutants T45A and N54A appeared to have an increased affinity for Bob1 compared with wild-type POU protein. Mutations Q27E, L53A, L55A and N59A do not efficiently supershift with Bob1. Mutants L53A and N59A are affected most dramatically in complex formation; both show much weaker interaction with Bob1 yielding in only 11% and 6% of wild-type supershifts, respectively. L55A was also affected, although to a lesser extent (28% compared with wild type). While these POU mutants have an affinity for the octamer site similar to wild-type POU, Q27E is difficult to interpret because it also shows a strongly reduced affinity to DNA (van Leeuwen *et al.*, 1995). The homeodomain mutants R13A+E17A, Q24A and T32A+D36A tested did not affect the interaction with Bob1 (Figure 3b). Figure 3c summarizes the known contacts of the coactivators VP16 (Lai *et al.*, 1992; Pomerantz *et al.*, 1992) and Bob1 with the Oct-1 POU domain. So far, only Bob1 seems to require residues in the POU-specific domain, whereas VP16 contacts the POU homeodomain. As illustrated in Figure 3d, L53 is part of the DNA recognition helix (helix 3), whereas L55 and N59 are located between this helix (helix 3) and helix 4. In particular, L55 and N59 are interesting because they seem to contact the fifth base pair of the octamer motif ATGCAAAT. As addressed below, this may have implications for the DNA binding behaviour of the Oct–Bob1 complex compared with free Oct factors.

Bob1 complex formation with Oct-1 or Oct-2 is dependent on the octamer sequence

Members of the POU family of transcription factors show a remarkable degree of flexibility of sequence recognition (Herr and Cleary, 1995). In the case of Oct-1 and Oct-2A, a variety of possible binding sites have been described in the regulatory sequences of ubiquitously expressed as well as cell type-specific genes. The observed B cell-specific activation of promoters containing the octamer motif can be explained by the combinatorial expression of Oct-1 or Oct-2A and their coactivator Bob1. However, if we assume that Bob1 would act as a cell type-specific amplifier of Oct factor-dependent transcription, the observation that a number of house-keeping gene promoters, like the histone H2B promoter, contain octamer sites but are obviously not expressed in a cell type-specific manner still remains paradoxical. While it remains possible that cell type-

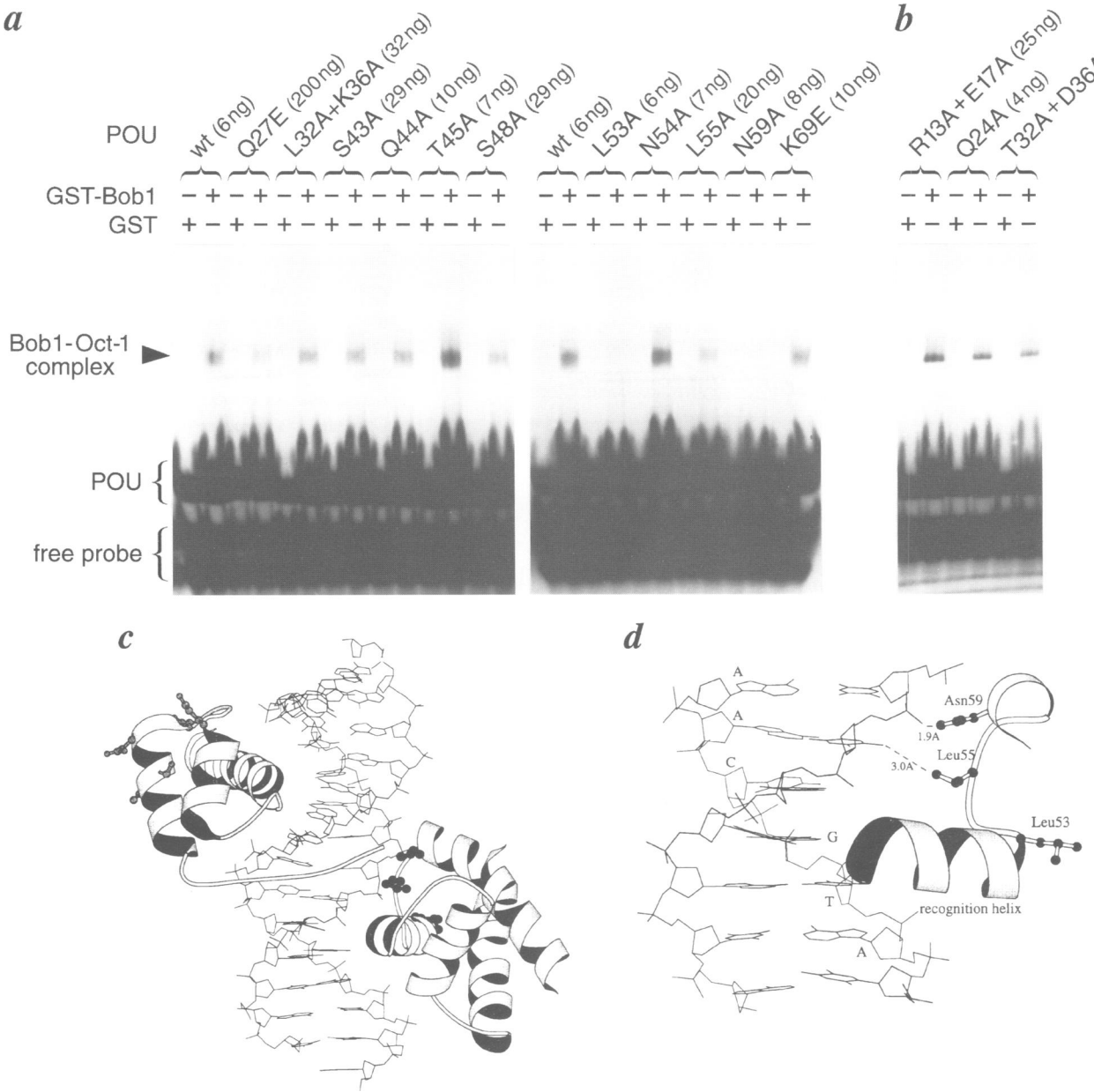

Fig. 3. Mutational analysis of the Bob1–Oct-1 interaction. (**a**) Bandshift assay with different POU-specific domain mutants and GST–Bob1 fusion protein expressed in *Escherichia coli*. 100 ng GST–Bob1 fusion protein or GST protein alone were preincubated with the indicated Oct-1 POU domain mutants for 20 min at room temperature, followed by another 20 min incubation at room temperature in the presence of the radiolabelled Igκ oligonucleotide. Bandshift activities of the various POU mutant proteins were adjusted in each bandshift reaction by the addition of different protein amounts, as indicated. (**b**) Analysis of different POU homeodomain mutants for ternary complex formation with Bob1. (**c**) Model of the Oct-1 POU domain. Residues crucial for interaction with VP16 (left side) and Bob1 (right side) are indicated as grey and black ball-and-stick models, respectively. (**d**) Location of residues within the POU-specific domain of Oct-1 found to be crucial for interaction with Bob1. Pictures were generated using the Molscript program (Kraulis, 1991), and the coordinates are from the Oct-1 POU–DNA crystal structure (Klemm *et al.*, 1994).

specific activation might require multiple protein–protein interactions of DNA-bound factors in a given promoter context, it is equally possible that the interaction of Bob1 with the POU domains of Oct-1 or Oct-2 could change their DNA binding behaviour and thus activate a subset of octamer promoters in a B cell-specific manner. To test this possibility, we assayed Oct–Bob1 complex formation in supershift experiments on several octamer variants from cellular and viral regulatory sequences known to be bound by free Oct-1/Oct-2A factors (Figure 4a).

Interestingly, only a subset of the cellular octamer variants, which are all recognized by Oct-1, are also bound by the Oct-1–Bob1 complex (Figure 4b). This is also the case when several octamer variants from viral promoter/enhancer sequences were tested. So far, only the octamer sites from the promoters of the Igκ (Kemler *et al.*, 1991), the proximal octamer site from the interleukin (IL)-2 promoter (Kamps *et al.*, 1990), the octamer sites from the H2B gene (LaBella *et al.*, 1988) and those from the SV40 enhancer (Schirm *et al.*, 1987) allowed ternary complex formation. All the other octamer variants, including the octamer from the CD20 promoter (Thevenin *et al.*, 1993),

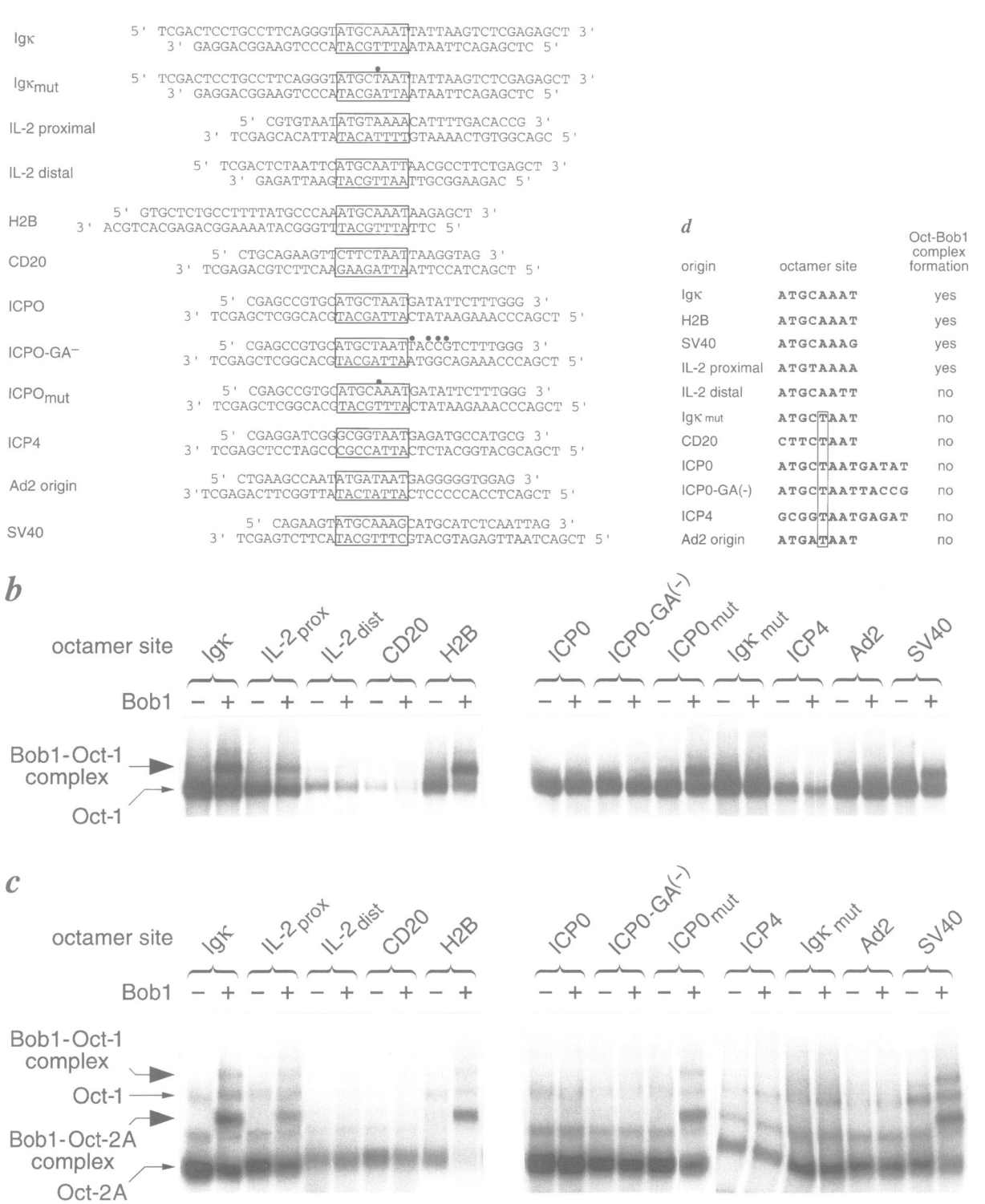

Fig. 4. Bob1-induced ternary complex formation with Oct factors is highly sequence specific. (**a**) Oligonucleotides used for the supershift experiments. (**b**) Ternary complex formation of Oct-1–Bob1 complexes on various octamer-containing oligonucleotides. Nuclear extracts from untransfected 293T cells and 293T cells transfected with 5 μg expression vector pCATCHNLS–Bob1 were tested for complex formation with the indicated oligonucleotides, as described previously. (**c**) Ternary complex formation of Oct-2A–Bob1 on various octamer oligonucleotides. Nuclear extracts from 293T cells transfected with 1 μg pCMV–Oct-2A alone (–) or together with 5 μg pCATCHNLS–Bob1 (+) were analysed for ternary complex formation as in (b). (**d**) Summary of the results from (b) and (c).

the IL-2 distal octamer (Kamps *et al.*, 1990), the viral motifs from herpes simplex virus immediate early genes ICP0 and ICP4 (O'Hare and Goding, 1988), and from the replication origin of the adenovirus type 2 (Pruijn *et al.*, 1988), did not allow binding of the Oct-1–Bob1 complex in our bandshift assay. In the case of the octamer from the ICP0 promoter, which is not bound by the Oct-1–Bob1 complex, only a single nucleotide at the fifth position (A to T) within the octamer consensus sequence is altered when compared with the octamer site of the Igκ which is

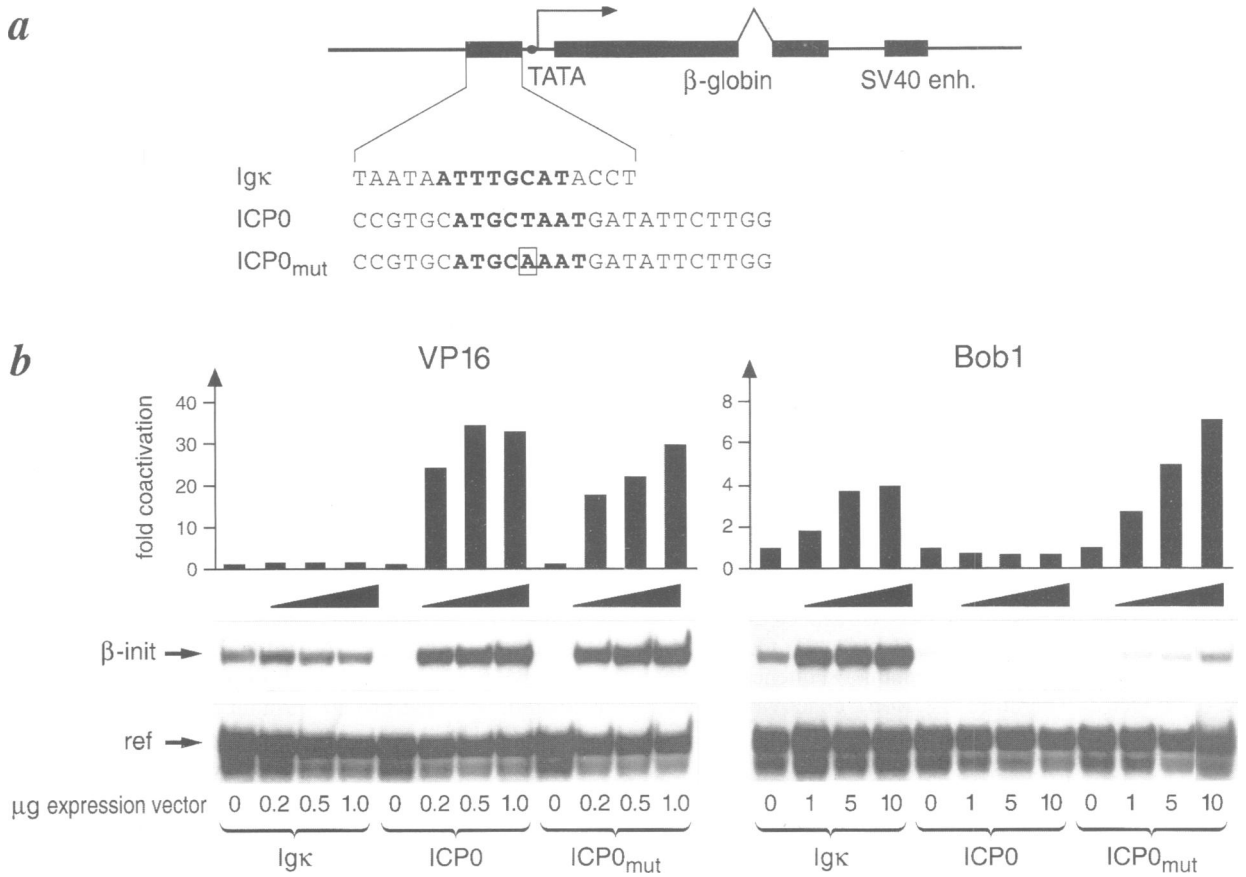

Fig. 5. Differential coactivation of Oct-1-dependent transcription from different promoters by VP16 and Bob1. (**a**) Schematic representation of the reporter genes used in the transfection. (**b**) S1 nuclease assay of HeLa cells transfected with 5 μg reporter, 0.5 μg reference gene CMV–REF and the indicated amounts of expression vector for VP16 (pGCNVP16) and Bob1 (pCATCHNLS–Bob1). For a graphic display of coactivation, the level of reporter gene transcription in the absence of Bob1 was taken as 1, irrespective of the absolute transcription efficiency.

bound by the complex. The GARAT sequence flanking the ICP0 octamer, which is known to be important for the interaction of Oct-1 with the herpes virus coactivator VP16, does not prevent ternary complex formation because removal of the GARAT portion from the ICP0 octamer motif did not restore binding of the Oct–Bob1 complex. However, binding of Oct-1–Bob1 complex was restored when the ICP0 octamer site was mutated at the fifth position (T to A), to generate a site identical to the Igκ octamer site. This result was confirmed with the reverse mutation within the Igκ octamer site, to make it identical to the ICP0 octamer which prevented binding of the Oct-1–Bob1 complex. An identical preference of Bob1 ternary complex formation with a subset of octamer motifs was found for Oct-2A (Figure 4c).

When we compared the octamer sequences that do not allow ternary complex formation, all of them, except the octamer of the IL-2 distal octamer site, contained a thymidine at the fifth position of the octamer motif (Figure 4d). All the motifs allowing ternary complex formation have a relatively intact ATGC 5′-half site in common, and always an adenosine at the fifth position, as found in the octamer consensus sequence. Taken together, these results show that the Oct–Bob1 complex shows a remarkably increased selectivity in DNA binding compared with free Oct-1 or Oct-2A. This indicates that the Bob1 coactivator does not simply act as an amplifier of Oct factor-dependent transcription, but also specifies its function through selective ternary complex formation to a subset of octamer-containing promoters.

Differential modulation of transcriptional activity of Oct-1 by the association with B cell cofactor Bob1 or herpes virus VP16

The data presented so far support the idea that transcription mediated by the DNA binding transcription factor Oct-1 is modulated not only quantitatively, by the recruitment of the Bob1 activation domain, but also qualitatively, through an altered DNA binding preference of the complex compared with Oct-1 alone. To test this idea, we analysed promoters containing the octamer sites of the ICP0 promoter, a mutant ICP0 octamer that allowed complex formation and the Igκ octamer site for Oct-dependent coactivation mediated by VP16 and Bob1 in HeLa cells (Figure 5b). VP16 coactivation, as expected, does not work from the Igκ promoter because it would require an additional GARAT sequence at the 3′ end of the octamer motif, as is present in the ICP0 promoter. Mutation of the ICP0 octamer motif ATGCTAAT to ATGCAAAT results in a similar coactivation by VP16. However, Bob1 coactivation is different in that it stimulates 4-fold the activity of a promoter containing an Igκ octamer site, while no activation is observed with the ICP0 TAATGARAT octamer site. However, if the ICP0 octamer site is mutated to be made identical to the Igκ octamer, the coactivation by Bob1 is restored. The overall activity on

the mutated ICP0 octamer is weaker than in the case of the Igκ octamer, which might be a result of differences in the flanking sequences, but the level of induced activation is similar.

These data demonstrate how subtle changes within the octamer motif can affect Oct-1–Bob1 ternary complex formation, leading to the selective activation of certain octamer promoters. Comparison of Bob1 with VP16 shows how the presence of different coactivators of octamer factors, which are unable to bind DNA on their own, differentially affect various octamer promoters.

Discussion

Recently we and others have described the isolation of a B cell-specific transcriptional coactivator for octamer binding transcription factors Oct-1 and Oct-2, variously termed Bob1, OCA-B and OBF-1 (Gstaiger *et al.*, 1995; Luo and Roeder, 1995; Strubin *et al.*, 1995). Here we report the structural basis of Oct–Bob1 ternary complex formation on octamer binding sites. Unexpectedly for a protein that cannot bind DNA on its own, the Oct–Bob1 complex is not formed with every octamer sequence bound by Oct-1 or Oct-2. Therefore the Bob1 coactivator does not simply act as a cell type-specific amplifier of Oct factor activity but rather targets its transactivation capacity to a subset of specific octamer sites in a promoter. This indicates a new level of selectivity in the regulation of octamer-containing promoters, which might further our understanding of promoter specificity.

Bob1 contains an interaction and a transactivation domain

In analogy to DNA-bound transcription factors which are, in most cases, organized into functionally separable modules, including DNA binding and transactivation domains, Bob1 consists of an N-terminal Oct-1 interaction domain and a C-terminal transactivation domain. Separate modules for contacting Oct-1 and a transactivation domain have also been described for the coactivator VP16 from herpes simplex virus (Triezenberg *et al.*, 1988). For Bob1, we found that a relatively short stretch, consisting of the first 65 amino acids, is sufficient for interaction with Oct factors. We mapped this interaction domain more precisely and found residues within a stretch from amino acids 26 to 32 to be crucial for contacting Oct-1. Secondary structure predictions of Bob1 using the PHD server (Rost *et al.*, 1994) resolved a helical region between amino acids 27 and 36, and also between amino acids 75 and 87. Interestingly, the amino acid stretch crucial for interaction maps to the first predicted helical region. A helical structure was also proposed for the part of the viral transactivator VP16 that interacts with Oct-1 (Greaves and O'Hare, 1990; Stern and Herr, 1991). In a deletion analysis, we have shown that coactivation requires a transcriptional activation domain located C-terminal to the interaction domain, which also confers transcriptional activity when fused to the heterologous DNA binding domain of GAL4. Most of the transactivation activity resides within the N-terminal half (amino acids 65–122) of the transactivation domain. Apart from a high number of prolines, a feature found in several other activation domains (Seipel *et al.*, 1992), an area of sequence homology to human NFAT4, SNF2a and E2F-2 was found in this region (Figure 1c), but the functional significance of this similarity is not clear at present. The existence of two separable functional domains favours the hypothesis that Bob1 coactivation works by simply tethering an additional activation domain to the Oct factor by its interaction domain. However, we cannot exclude the alternative possibility, namely that Bob1 could also unfold a cryptic activation domain in the Oct factor itself. Because the interaction domain of Bob1 alone is unable to boost Oct factor-dependent transcription, such an allosteric function would have to overlap with the transactivation function.

The Oct–Bob1 interaction provides a new mechanism in the selective activation of octamer-containing promoters

The bipartite structure of POU domain proteins is believed to be responsible for the flexibility in target site recognition by the POU domain proteins (Herr and Cleary, 1995). Given the great number of possible binding sites in a mammalian genome, the flexibility in DNA binding of POU factors and other DNA binding transcription factors raises the question as to how a promoter can be selectively activated. Multiple protein–protein interactions were proposed to play a major role in the specificity of POU protein function (Rosenfeld, 1991; Herr and Cleary, 1995). Several DNA binding transcription factors, like Sp1, AP-1 and PR/GR, can bind DNA cooperatively through association with POU proteins. Only two proteins, namely VP16 from herpes simplex virus and B cell-specific Bob1, are known to interact with the Oct-1 POU domain but cannot bind DNA in the absence of Oct-1. They would therefore be defined as true coactivators (Herr and Cleary, 1995).

The finding that the Bob1 coactivation from the octamer-containing promoter of the ubiquitously expressed H2B gene could not be detected *in vitro* or is reduced compared with the Igκ promoter when assayed in HeLa transfection experiments *in vivo* (Luo and Roeder, 1995; Strubin *et al.*, 1995) already provides evidence for promoter-specific coactivation. At present, the reduced coactivation of the H2B octamer promoter is difficult to explain mechanistically because Bob1 and Oct-1 are able to form a ternary complex with the H2B octamer sequence identical to the Igκ octamer site (Figure 4b). Perhaps the architecture of the H2B promoter involving additional DNA binding factors (Hinkley and Perry, 1992) and their cognate coactivators, together with their correct spacing to the TATA-box, determines the assembly of a specific general transcription factor complex, regardless of whether or not Bob1 is bound to the Oct factor. We tested one possible aspect of the H2B architecture, namely the spacing between the octamer site and the TATA-box which is conserved in several H2B genes; however, we could not find a significant influence on Bob1 coactivation (our unpublished results). Therefore we think that the other features of the promoter context of the H2B gene play a critical role in determining the level of Bob1 coactivation.

In this study we focused on an additional and more direct mechanism by which the Bob1–Oct interaction could help to understand the specification of octamer promoters. As Bob1 is capable of specifically interacting with the DNA binding domain of Oct-1 and Oct-2, we

asked whether the Oct–Bob1 complex would preferentially activate a subset of octamer promoters because of differential ternary complex formation. When analysed in more detail, the Oct–Bob1 interaction was found to require residues located in the POU-specific domain of Oct-1, in contrast to pTP–Pol, HMG2 and VP16 which need the POU homeodomain of Oct-1 for interaction (Stern and Herr, 1991; Coenjaerts *et al.*, 1994; Zwilling *et al.*, 1995). However, as we have analysed a limited number of POU homeodomain mutants, we cannot exclude the possibility that additional contacts located in the homeodomain or even outside it might also play a role in the association with Bob1. Preliminary experiments, where we have tested a protein consisting of the POU-specific domain of Oct-2 fused to the antennapedia homeodomain of *Drosophila* (Brugnera *et al.*, 1992), indeed indicate that the POU-specific domain is insufficient for the association with Bob1 (our unpublished results). Remarkably, the point mutations within the POU-specific domain that reduce complex formation with Bob1 involve residue L53, which is part of the DNA recognition helix (helix 3), and residue L55, as well as residue N59 located between helices 3 and 4. As illustrated in Figure 3d, L55 and N59 seem to contact the fifth base pair of the octamer motif ATGCAAAT. Therefore we think that an interaction of Bob1 with this region may influence the DNA binding behaviour of the Oct–Bob1 complex compared with free Oct factor. This is in good agreement with what we found when comparing the binding preference of the Oct–Bob1 complex with that of free Oct-1 or Oct-2 factor: the fifth base pair within the octamer consensus motif appears important for ternary complex formation, as it is also the same base pair that is contacted by the residues important for Bob1 binding. This is the case for the octamer sites from the ICP0, ICP4 and CD20 promoters and the Ad2 origin of DNA replication that fail to form a ternary complex with Oct-1 and Bob1 and have a thymidine substitution at the fifth position. The finding that mutations at the fifth position of the octamer motif, introduced in the ICP0 promoter site (ATGCTAAT to ATGCAAAT), can restore complex formation, while in the Igκ element an ATGCAAAT to ATGCTAAT mutation prevents complex formation, underline the importance of this base pair for ternary complex formation. The only exception is given by the inability of the IL-2 distal weak affinity octamer site ATGCAATT (Kamps *et al.*, 1990) to support Oct-1–Bob1 complex formation. This indicates that Bob1 binding to DNA-associated Oct can depend on other structural features too. Our coactivation studies show that slight structural alterations within or flanking the octamer motif can be sensed based on protein–protein interactions between Oct-1 and Bob1. In contrast to Bob1, VP16–Oct-1 complex formation and transcriptional activation are certainly possible with both the ATGCAAAT and ATGCTAAT octamer sequences as long as they contain a 3′ extension known as the GARAT motif, as found in the viral ICP0 and ICP4 promoters (Walker *et al.*, 1994; Douville *et al.*, 1995).

A model for selective Oct–Bob1 ternary complex formation

Two possibilities can be envisaged regarding the mode of ternary complex formation between Bob1, Oct-1/Oct-2 and the octamer site. First, Bob1 forms a stable complex with Oct factors in the absence of DNA, and this complex recognizes only a subset of octamer sites. Second, Bob1 detects specific conformations of the Oct–DNA complexes induced by different octamer sites. Recent studies indicate that Oct-1 can indeed adopt alternate conformations when bound to different octamer sites, which is believed to be the structural basis for selective complex formation with VP16 (Walker *et al.*, 1994; Cleary and Herr, 1995). In the case of Bob1, a protein–protein interaction with purified Oct-1 POU protein in the absence of DNA has been reported (Luo and Roeder, 1995). However, our order of addition experiments suggest that such free Oct–Bob1 complexes are considerably less stable than the ternary complex with DNA (our unpublished results). From this, we conclude that Bob1 binds the Oct-1–DNA complex with much higher affinity than in the absence of DNA. This favours the second hypothesis, namely that selectivity is based on the recognition of structural features of Oct-1 bound to the one or other type of octamer sequence.

The results described here indicate that the conformational flexibility in DNA binding by POU proteins provides the basis for the differential interaction with specific coactivators, a mechanism contributing to cell type-specific gene activation.

Materials and methods

Construction of expression vectors

All recombinant plasmids were generated using standard techniques for recombinant DNA work (Maniatis *et al.*, 1989). Details of the constructions are available on request. C-terminal Bob1 deletion mutants Bob1(1–65), Bob1(1–122) and Bob1(1–192) were expressed from a cytomegalovirus (CMV)-based vector pCATCHNLS (Georgiev *et al.*, 1996) by inserting the restriction fragments obtained from a digest of pCATCHNLS–Bob1 (Gstaiger *et al.*, 1995) with *Ava*II, *Afl*III or *Rsa*I, respectively, together with *Bam*HI and cloning into a *Bam*HI and a blunt-ended *Xba*I site of pCATCHNLS. N-terminal deletion mutants Bob1(65–256), Bob1(122–256) and Bob1(193–256) were obtained by cutting out fragments from pCATCHNLS–Bob1 with *Ava*II, *Afl*III or *Rsa*I together with *Xba*I and inserting them back into the pCATCH. Reporter plasmids ICP0–OVECS and ICP0mut–OVECS were generated by cloning the respective double-stranded oligonucleotides into the *Sac*I–*Sal*I sites of the β-globine reporter plasmid OVECS (Westin *et al.*, 1987).

Generation of Bob1 point mutations

For mutagenesis of the interaction region, we used a novel PCR-based two-hybrid selection system in yeast, as described previously (Gstaiger *et al.*, 1996). A region including the first 438 bp of the Bob1 cDNA, isolated as a plasmid (pACT–Bob1) from the initial two-hybrid screen, was amplified with PCR under degenerative conditions (1 mM dNTP, 500 fm primer, 200 ng pACT–Bob1 template DNA, 0.45 mM $MgCl_2$, 0.1 mM Tris–HCl, pH 9.0, 0.1% Triton, 0.01% gelatine, 50 mM KCl; hot start, 1 min at 85°C followed by two rounds of 40 cycles of 45 s at 94°C, 30 s at 50°C, 3 min at 72°C; final elongation for 7 min at 72°C) using primers specific for the N-terminal GAL4 acidic activation domain (5′-TGTTTAATACCACTACAATGG-3′) and for the region at amino acid 121 of the Bob1 (5′-ACATACATGTCAGCTGAGTA-3′) cDNA. For generating yeast vectors expressing mutant Bob1 proteins, mutated PCR fragments were transformed together with the *Bam*HI-linearized vector pACT–Bob1, as described previously (Gietz, 1992) into yeast strain RH6IIE expressing Oct-2A. After recombination of the PCR products with the vector pACT–Bob1, interaction mutants were selected simply by the inability to induce the *lacZ* reporter gene through their loss of interaction with Oct-2A and were analysed by sequencing. Vectors for the expression of mutant Bob1 proteins in mammalian cells were generated by transferring mutant Bob1 cDNAs obtained from the yeast screen as *Bam*HI fragments into pCATCHNLS.

Nuclear extract preparation and gel retardation assay

Nuclear extracts from 293T and HeLa cells contained 1–3 μg/μl total protein and were prepared as described previously (Schreiber *et al.*, 1989). For protein expression in rabbit reticulocyte lysates, pCATCH-based constructs were used for the *in vitro* transcription of mutant or wild-type Bob1 cDNA. *In vitro* translation was performed according to the instructions of the manufacturer (Promega). For antibody supershift experiments, HeLa nuclear extracts (1 μl) were incubated with 4 μl reticulocyte lysate-translated Bob1 proteins and 2 μl monoclonal anti-FLAG antibody (M2, Kodak) for 15 min at room temperature in bandshift buffer containing 10–20 fmol of the respective ^{32}P-labelled DNA probe; they were analysed by gel electrophoresis, as described previously (Gstaiger *et al.*, 1995). In all other gel retardation experiments shown, the bandshift reaction was performed in electrophoretic mobility shift assay buffer (4% Ficoll, 20 mM HEPES, pH 7.9, 50 mM KCl, 1 mM dithiothreitol and 0.25 mg/ml bovine serum albumin) containing 0.4 μg poly d(I)d(C) and 0.1 μg herring sperm DNA per reaction in a total volume of 20 μl. For expression in bacteria, Bob1 cDNA was cloned into pGEX2T (Pharmacia); glutathione-S transferase (GST)–Bob1 fusion protein was purified using GST beads. The expression and purification of Oct-1 POU mutant proteins have been described elsewhere (van Leeuwen *et al.*, 1995). The quantitation of supershift band intensities of various Oct-1 POU mutants was performed by X-ray film densitometry (Molecular Dynamics). Values given in the text are corrected for DNA binding activity and represent the percentage of ternary complex formation relative to the wild-type POU protein.

Expression in mammalian cells and S1 nuclease assay

HeLa and 293T cells were transfected by the calcium phosphate coprecipitation method. For RNA mapping experiments and nuclear extract preparation, cells were harvested 36 h after transfection. Nuclear extract preparation, RNA isolation and S1 nuclease mapping were performed as described previously (Gstaiger *et al.*, 1995). Autoradiographs were quantified with a phosphorimaging device (Molecular Dynamics), and the signals of the reporter genes were normalized to reference signals to correct for the variability in transfection efficiency.

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